Cooperative Antitumor Effect of Multitargeted Kinase Inhibitor ZD6474 and Ionizing Radiation in Glioblastoma

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Abstract

Purpose: Glioblastoma multiforme is an aggressive disease in which vascular endothelial growth factor (VEGF) and the EGF receptor (EGFR) are implicated in tumor growth, relapse, and resistance to radiotherapy and chemotherapy. The VEGF receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR), typically present on endothelial cells, have also been identified in human glioblastoma tissues and cell lines. In addition, EGFR is dysregulated in the majority of human glioblastomas and EGFR overexpression correlates with shorter survival. We have investigated the antitumor and antiangiogenic effect of ZD6474, an inhibitor of both VEGFR and EGFR signaling as a single agent and in combination with ionizing radiation.

Experimental Design: We have used ZD6474 and/or ionizing radiation in human glioblastoma cell lines D54 and U251 in vitro and in nude mice bearing established xenografts. The effects of treatment on tumor blood vessels and protein expression were evaluated by Western blot and immunohistochemistry.

Results: As single agents, ionizing radiation and ZD6474 caused a dose-dependent inhibition of soft agar growth in D54 and U251 cell lines, whereas a cooperative effect was obtained in combination. Treatment of mice bearing D54 xenografts with either ZD6474 or radiotherapy alone caused tumor growth inhibition that was reversible upon treatment cessation. A cooperative and long-lasting inhibition of tumor growth was obtained with ZD6474 in combination with concomitant radiotherapy. The antiproliferative effect was accompanied by inhibition of VEGF protein expression and inhibition of angiogenesis as measured by vessel counting.

Conclusion: This study shows the antitumor activity of ZD6474 in combination with ionizing radiation in glioblastoma both in vitro and in vivo, and provides a scientific rationale to evaluate ZD6474 alone or in combination with radiotherapy in patients affected by this disease.

Glioblastoma multiforme is characterized by high proliferative rate and vascularization and, in spite of the improvements obtained with radiotherapy and chemotherapy, is associated with a poor outcome. The vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) seem to play a major role in contributing to growth and relapse of glioblastomas and have also been implicated in the disease resistance to radiotherapy and chemotherapy (1, 2).

VEGF is the most important and best-characterized angiogenic factor and induces a potent and specific mitogenic signal for endothelial cells by binding to endothelial cell receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR). Expression of VEGF has been associated with more aggressive disease and poorer outcome in different types of cancer (3–5). Activation of EGFR signaling can up-regulate the production of VEGF in human cancer cells and we and others have provided evidence that EGFR blockade causes inhibition of the secretion of VEGF and of other angiogenic growth factors (6). Effective blockade of the VEGF pathway has been achieved with different agents (4). ZD6474, an orally available inhibitor of VEGFR-2 tyrosine kinase, which is also able to inhibit EGFR and RET tyrosine kinase activity, has been shown to have antitumor activity against a broad spectrum of histologically diverse human tumor xenografts (7–9). ZD6474 can cooperate with taxanes in nude mice bearing human tumors, causing a marked antiproliferative and antiangiogenic activity (8). Clinical evaluation of ZD6474 in cancer patients is currently ongoing (10).
Glioblastoma frequently exhibit an overexpression of EGFR, sometimes present as the truncated isoform EGFRvIII, which has been implicated in relapse and poor prognosis of this disease (2, 11). Nevertheless, targeting EGFR signaling after failure of chemotherapy or radiotherapy shows only moderate activity (12). It has been hypothesized that this may occur because both EGFR-dependent and VEGF-dependent stimuli act together to cooperatively induce the recovery of quiescent glioblastoma cells surviving chemotherapy or radiotherapy, thereby promoting disease relapse. Interestingly, glioblastoma cells express VEGFRs, perhaps suggesting an angiogenesis-independent role for the VEGF-signaling pathway in disease progression in glioblastoma (13).

We have previously shown that combination of radiotherapy with a small-molecule inhibitor of EGFR tyrosine kinase, or a monoclonal antibody (mAb) directed against EGFR, produces a cooperative effect in different human tumor models (14, 15). On the other hand, we and others have shown that over-expression of VEGF is a major escape pathway following efficient EGFR blockade (16, 17) and that ZD6474, by simultaneously blocking EGFR and VEGF-R2, can control tumor growth in tumor cells resistant to selective anti-EGFR drugs (17).

In this study, we have, therefore, evaluated response to ZD6474 therapy, alone and in combination with radiotherapy, in glioblastoma cells in vitro and in vivo. We have shown that (a) ZD6474 inhibits the in vitro growth of glioblastoma cell lines that express both functional EGFR and VEGF-R2; (b) ZD6474 potentiates the antitumor activity of radiotherapy in vitro and in vivo in s.c. model systems of human glioblastoma; and (c) the potent inhibitory effect is accompanied by modulation of expression of proteins critical for tumor growth, apoptosis, and angiogenesis, as evaluated by biochemical and histochemical analysis of tumor specimens.

**Materials and Methods**

**Drugs.** ZD6474 was kindly provided by AstraZeneca (Macclesfield, United Kingdom).

**Cell lines.** Human D54 and U251 glioblastoma cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in DMEM supplemented with 10% fetal bovine serum, 20 mmol/L HEPES (pH 7.4), penicillin (100 IU/mL), streptomycin (100 μg/mL), and 4 mmol/L glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

**Western blot analysis.** For the assessment of EGFR expression and phosphorylation, total cell protein extracts were obtained, as previously described (17), from D54 and U251 cells. Total cell lysates were resolved by 7.5% SDS-PAGE and probed with either an antihuman EGFR mAb (Transduction Laboratories, Lexington, KY), the VEGFR-1 mAb and the VEGFR-2/KDR mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and polyclonal anti-RET antibody (9). For evaluation of the expression of different proteins in D54 tumor xenografts, two tumors from each group of mice were collected and lysed using a dounce tissue homogenizer; total cell protein extracts were resolved by a 4% to 20% SDS-PAGE and probed with anti-human VEGF mouse mAb (Santa Cruz Biotechnology) or anti–bcl-2 rabbit polyclonal antibody (Transduction Laboratories); immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, London, United Kingdom) as described previously (17).

**Ionizing radiation and ZD6474 treatment and growth in soft agar.** Exponentially growing cells were irradiated in 100-mm tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) using a 6 MV photon linear accelerator (General Electric, Buckinghamshire, United Kingdom). After irradiation, cells were trypsinized and 10⁴ cells/well were suspended in 0.5 mL of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 mL of 0.8% agar medium base layer in 24-multwell cluster dishes (Becton Dickinson) and treated, in the combination treatment experiments, every day for a total of 4 days with different concentrations of ZD6474. In the experiments in which cancer cells were treated after 10 to 14 days, cells were stained with nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO) and colonies >0.05 mm were scored.

**Tumor xenografts in nude mice.** Four- to 6-week-old athymic BALB/c nu/nu female mice were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance with the institutional guidelines of the University of Naples Federico II Animal Care and Use Committee. Mice were acclimatized at the University of Naples Federico II Medical School Animal Facility for 1 week before receiving injections of cancer cells. Mice were injected s.c. with 10⁷ D54 or U251 cells that had been resuspended in 200 μL of Matrigel (Collaborative Biomedical Products, Bedford, MA). After 7 days, when established tumors of 0.1 cm³ volume were detected, mice were randomized to receive i.p. administration of ZD6474 (50, 75, or 100 mg/kg/d on days 1-5 of each week for 3 weeks) and/or radiotherapy (2 Gy/dose daily, on days 2 and 4 of each week for 2 weeks; in those days, radiotherapy was administered 2 hours after ZD6474 treatment). For each experiment, treatment groups comprised eight mice. Tumor volume was measured using the following formula: \( \pi \left( \frac{a}{2} \right) \times \left( \frac{b}{2} \right) \times \left( \frac{c}{2} \right) \times \left( \frac{d}{2} \right) \). In the experiments in which cancer cells were treated after 10 to 14 days, cells were stained with nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO) and colonies >0.05 mm were scored.

**Immunohistochemical analysis.** Immunocytochemistry was done on formalin-fixed, paraffin-embedded tissue sections (5 μm) of D54 xenografts as previously reported (15). After overnight incubation with the appropriate primary antibody at 4°C, sections were washed and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution, Vectastain ABC kit; Vector Laboratory, Burlingame, CA), washed again, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide as described previously (15). The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. An anti-Ki67 mAb (clone MIB1; DBA, Milan, Italy) was used at 1:100 dilution. To determine the percentage of positive cells, at least 1,000 cancer cells per slide were counted and scored (15). New blood vessels were measured using a cell counting software program (Image Tool version 3.0, University of Texas Health Science Center, San Antonio, TX). The number of blood vessels was determined by counting vessels that had a diameter of 10–100 μm using the same software. For each field, the number of stained blood vessels was counted. For individual tumors, microvessel count was scored by averaging the five field counts (15).

**Statistical analysis.** The Student’s t test was used to evaluate the statistical significance of the results. All P values represent two-sided tests of statistical significance. Analyses were done with the program New System statistical package for XP (BMDP Statistical Software, Los Angeles, CA).

**Results**

**Protein expression and inhibition of cell growth in soft agar.** Because ZD6474 can potentially inhibit both VEGFR-2-dependent tumor angiogenesis and EGFR-dependent pathways within tumors, we first analyzed by Western blot the expression of EGFR, VEGFR-1, and VEGFR-2. U251 cells express higher levels of EGFR than D54, whereas VEGFR-1 and VEGFR-2 were detectable in both cell lines (Fig. 1A). Conversely, no expression of RET was detected (data not shown). We then evaluated the effects of ZD6474 or ionizing radiation on the
soft agar cloning formation in efficiency of D54 and U251 human glioblastoma cancer cell lines. Ionizing radiation treatment caused a dose-dependent inhibition in soft agar growth in the two cell lines, with an IC\textsubscript{50} of 45 cGy for the D54 cells and 15 cGy for the more sensitive U251 cells, as shown in Fig. 1B. Treatment with ZD6474 resulted in a dose-dependent inhibition of colony formation of both cell lines with an IC\textsubscript{50} of 5 \textmu mol/L for the D54 and a very low IC\textsubscript{50} of 0.05 \textmu mol/L for the U251 cells (Fig. 1C).

We next examined whether the combined treatment with ZD6474 and ionizing radiation could provide any cooperative antiproliferative effect. In both cell lines, the combined treatment caused a potent inhibition of cell growth with all doses of ZD6474 and ionizing radiation used. In D54 cells, single treatment with ZD6474 0.1 \textmu mol/L or ionizing radiation 25 cGy caused an average 15% to 20% growth inhibition, whereas the combination of the two treatments caused a 50% inhibition of colony formation in soft agar (Fig. 2A). Similarly, the combined treatment greatly enhanced the growth inhibitory effect obtained with each single agent in U251 cells (Fig. 2B).

**Inhibition of xenograft growth in nude mice.** We evaluated the effects induced by ionizing radiation and ZD6474 treatment in an \textit{in vivo} model by establishing s.c. tumors from
the two glioblastoma cell lines in immunodeficient mice. When D54 or U251 tumors were established, 7 days after tumor cell injection, the nude mice were treated i.p. for 5 d/wk with ZD6474, 50 or 100 mg/kg for 3 weeks. As illustrated in Fig. 3A and B, 50 days after tumor cell injection treatment with ZD6474 for 3 weeks caused an inhibition of tumor growth in all the mice. To test the effect of the combined treatment in vivo, we used the D54 tumor model, which is less sensitive to ZD6474. Seven days after tumor cell injection, nude mice with D54 xenografts of ~0.15 cm³ were treated with ZD6474 (75 mg/kg/dose i.p. for 5 d/wk for 3 weeks) and/or with ionizing radiation (2 Gy/dose twice a week, on days 2 and 4, for 2 weeks). As shown in Fig. 4A, mice in the control group had been sacrificed due to tumor burden by day 56, 8 weeks after tumor implantation. At the same time point, the tumor growth in mice treated with either ZD6474 or radiotherapy alone was markedly inhibited, but growth rate eventually recovered, reaching a mean tumor volume of >2.0 cm³ within 3 weeks. Treatment with ZD6474 plus radiotherapy caused a persistent inhibition of D54 tumor growth. At day 49, the tumor size was 2.3 cm³ ± SE in control animals, 1.07 cm³ ± SE after radiotherapy alone, 1.2 cm³ ± SE after ZD6474 alone, and 0.32 cm³ ± SE in the combination group. The Student’s t test, used to compare tumor volumes among different treatment groups and control untreated mice, showed tumor volumes significantly different compared with controls (P < 0.001 for each comparison). At this time point, the animals in the control group were sacrificed due to tumor burden. At day 63, 5 weeks after treatment withdrawal, the tumors in the combination therapy group were 70% smaller compared with those of mice treated with each agent alone. Moreover, at day 70, 6 weeks after treatment withdrawal, the mean tumor size in the combination therapy group was 0.98 cm³ ± SE, whereas animals in each of the single agent treatment groups had been sacrificed because the tumor volume was >2.0 cm³.

Inhibition of protein expression and angiogenesis in tumor samples. We did Western blot analysis on D54 tumor extracts from mice in all the treatment groups. Mice were injected s.c. in the dorsal flank with 10⁷ D54 cells suspended in 200 μL of Matrigel. After 7 days (average tumor size 0.1 cm³), mice were treated with ionizing radiation (2 Gy/dose twice a week for 2 weeks) alone, or with ZD6474 (75 mg/kg/dose i.p. for 5 d/wk for 3 weeks) alone, or with a combination of both. Each group consisted of 10 mice. Points, average; bars, SE. Student’s t test was used to compare tumor sizes among different groups at day 49 after a D54 cell injection. ZD6474 versus control (P < 0.001); radiotherapy plus ZD6474 versus control (P < 0.001). B, Western blot analysis of VEGF expression in human D54 cancer xenografts. Analysis was done on cell lysates from two tumors removed after 2 weeks of treatment on day 25. Lane 1, control, untreated tumors; lane 2, tumors from mice treated with radiotherapy; lane 3, tumors from mice treated with ZD6474; lane 4, tumors from mice treated with radiotherapy plus ZD6474.

Fig. 3. Antitumor activity of ZD6474 on established D54 (A) or U251 (B) human glioblastoma xenografts. Mice were injected s.c. in the dorsal flank with 10⁷ D54 cells suspended in 200 μL of Matrigel. After 7 days, mice were treated with ZD6474 alone on days 1 to 5 for 3 weeks. Each group consisted of 10 mice. Points, average; bars, SE.

Fig. 4. A, antitumor activity of ionizing radiation and ZD6474 on established D54 human glioblastoma xenografts. Mice were injected s.c. in the dorsal flank with 10⁷ D54 cells suspended in 200 μL of Matrigel. After 7 days (average tumor size 0.1 cm³), mice were treated with ionizing radiation (2 Gy/dose twice a week for 2 weeks) alone, or with ZD6474 (75 mg/kg/dose i.p. for 5 d/wk for 3 weeks) alone, or with a combination of both. Each group consisted of 10 mice. Points, average; bars, SE. Student’s t test was used to compare tumor sizes among different groups at day 49 after a D54 cell injection. ZD6474 versus control (P < 0.001); radiotherapy plus ZD6474 versus control (P < 0.001). B, Western blot analysis of VEGF expression in human D54 cancer xenografts. Analysis was done on cell lysates from two tumors removed after 2 weeks of treatment on day 25. Lane 1, control, untreated tumors; lane 2, tumors from mice treated with radiotherapy; lane 3, tumors from mice treated with ZD6474; lane 4, tumors from mice treated with radiotherapy plus ZD6474.
observed in all treatment conditions (data not shown). Immunohistochemical evaluation of D54 tumors cell growth, as assessed by Ki67/Mib-1 nuclear staining, confirmed a moderate inhibitory effect with ZD6474 or radiotherapy treatment (Table 1), whereas <5% of the cancer cells were Mib-1 positive in D54 tumors treated with the combination of ZD6474 plus radiotherapy. D54 vascularization was quantified by new microvessel count, using the anti–Factor VIII antibody. Radiotherapy or ZD6474 treatment alone reduced microvessel count by ~20% and 40%, respectively (Table 1). Combined treatment caused a 70% reduction of microvessel count and increased necrosis (Table 1).

Discussion

In the past 10 years, increasing experimental and clinical evidence has accumulated to support the development of antiangiogenic strategies for tumor therapy. In particular, VEGF and its receptors have been the focus of extensive studies (18).

In glioblastoma multiforme, both EGFR- and VEGFR-dependent signaling play a critical role in driving the growth and relapse of the disease. Previous studies have also suggested that high levels of EGFR in a tumor predict for intrinsically radioresistant and increased rates of disease relapse following therapy (19), demonstrating a positive correlation between high levels of EGFR expression and poor tumor radiation response (20). In turn, overexpression of VEGF in glioblastoma has been associated with increased vascular density and a poor clinical prognosis (13). Moreover, ionizing radiation–induced VEGF expression is thought to provide a positive survival signal, protecting the tumor endothelium from irradiation-induced cytotoxicity. It is notable that 80% of glioblastomas overexpress VEGF receptors, suggesting not only a paracrine but also a possible autocrine action of VEGF in this tumor setting (21–23). In addition, endothelial cells in the tumor neo-vascularature have been shown to overexpress EGFR or transforming growth factor α, as well as activated EGFR, potentially rendering the tumor vasculature sensitive to direct inhibition by EGFR antagonists (24). However, selective inhibitors of EGFR signaling used alone after failure of chemotherapy or radiotherapy have shown only moderate activity. Taken together, all these studies suggest that the cooperation between EGF- and VEGF-activated signaling represents a critical issue in glioblastoma and is, therefore, a potentially important therapeutic target. Based on this, we have evaluated whether the combination of radiotherapy with the simultaneous blockade of both EGF and VEGF signaling by ZD6474 may represent a valuable therapeutic approach against glioblastoma.

ZD6474 treatment causes a dose-dependent inhibition of cell growth in glioblastoma cell lines D54 and U251. However, these glioblastoma cell lines seem to have a different intrinsic sensitivity to inhibition of cell growth by ZD6474. The sensitivity of D54 cells falls within the range of IC50 values previously reported for other tumor cell lines (7), providing supporting evidence that in vivo antitumor activity of ZD6474 in the D54 tumor model is also through inhibition of angiogenesis. In contrast, U251 cells seem to be much more sensitive to growth inhibition by ZD6474, suggesting that in vivo effects in this tumor model may be a consequence of a combination of both direct antitumor effects and inhibition of angiogenesis. We have shown that both D54 and U251 cells express EGFR, VEGFR-1, and VEGFR-2, whereas they do not express RET, suggesting that the observed antiproliferative effect of ZD6474 in these cells may be attributable to the inhibition of EGFR- and/or VEGFR-dependent mitogenic signaling.

A recent study evaluated the effect of the combination of ZD6474 with radiotherapy in a non–small cell lung cancer model, analyzing the sequence of radiotherapy and ZD6474 administration (25). The better results were obtained using ZD6474 immediately after radiotherapy rather than concomitantly. In that study, ZD6474 alone did not have a significant effect on tumor vessel density, although it did reduce tumor perfusion. It was suggested that an increased hypoxic fraction resulting from reduced tumor perfusion may have contributed to the lower relative effect obtained when ZD6474 was given concomitantly with radiotherapy. On the other hand, an earlier study (26) had shown that a monoclonal antibody to VEGF reduced VEGF-dependent interstitial fluid pressure and hypoxia, favoring the effect of subsequent radiotherapy in tumors, including glioblastoma. In the present study, we used a concurrent combination dosing schedule, administering ZD6474 for 5 d/wk together with fractionated radiotherapy (2 Gy) on days 2 and 4 of each week.

In both glioblastoma cell lines, combined treatment with ZD6474 and ionizing radiation provided significant additional inhibition of cell growth in vitro with all doses of ZD6474 and ionizing radiation tested, compared with single-agent treatment alone. Because U251 showed hypersensitivity to ZD6474 treatment, we carried out experiments in nude mice using the D54 tumor model. Treatment of mice bearing established D54 xenografts with radiotherapy in combination with ZD6474 caused a persistent inhibition of growth, significantly increasing survival (as measured by time to reach mean tumor volume of >2.0 cm³) compared with single-agent treatment alone. This potent antitumor effect was accompanied, after 2 weeks of treatment, by a significant reduction in VEGF expression and a marked reduction in microvessel count within the D54 tumors. We also evaluated the bcl-2 levels in the D-54 tumor samples observing a moderate increase in all treatment groups, suggesting that the bcl-2–dependent pathway may not be directly involved in the observed antitumor effects.

Although best schedule(s) and sequence(s) for combining radiotherapy with selective inhibitors of angiogenesis have still to be identified, our preclinical study has shown that dual EGF and VEGF-2 tyrosine kinase inhibitor ZD6474 may have a valuable role in human glioblastoma and that combining

| Table 1. Immunohistochemical analysis of D54 glioblastoma xenografts after treatment with ZD6474 and radiotherapy |
|-------------------------------------------------|-----------------|-------------------|
| Factor VIII (vessels per high-power field)  | Mib-1 (%Mib1-positive cells) | Mib-1 intensity |
| Control                                      | 10              | 70%               | ++               |
| Radiotherapy                                 | 8               | 50%               | ++               |
| ZD6474                                       | 6               | 40%               | ++               |
| Radiotherapy + ZD6474                        | 3               | <5%               | +                |
ZD6474 with concurrent radiotherapy can produce significant cooperative antitumor effects in vitro and in vivo. ZD6474 is currently under clinical evaluation and has shown some evidence of antitumor activity in non-small cell lung cancer (27). The results of the present study, within the limits of this xenograft model, provide a scientific rationale for testing the combination of ionizing radiation with ZD6474 in patients with glioblastoma.

References
4. Kerbel R, Folkman J. Clinical translation of angiogen-
9. Wedge SR, Ogilvie DJ, Dukes M, et al. ZD6474 inhibits vascular endothelial growth factor signalling, angiogenesis, and tumor growth following oral admini-
10. Ciardiello F, Caputo R, Damiano V, et al. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth fac-
13. Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY. Epidermal growth factor stimulates vas-
cular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multi-
16. Asano M, Yukita A, Matsunuma T, Kondo S, Suzuki H. Inhibition of tumor growth and metastasis by an immu-
18. Millauer B, Shawver KK, Plate KH, Risau W, Ulrich A. Glioblastoma growth inhibited in vivo by a domi-
20. Williams KJ, Telfer BA, Brave S, et al. ZD6474, a potent inhibitor of vascular endothelial growth factor signaling, combined with radiotherapy: schedule-
21. Lee CG, Heijn M, Di Tomaso E, et al. Anti-Vascular endothelial growth factor receptor treatment augments tumor radiation response under normoxic or hypoxic condi-
22. Heymach JV, Dong RP, Dimery I, et al. ZD6474, a novel antiangiogenic agent, in combination with docetaxel in patients with NSCLC: results of the run-